Characterization of TRP-1 mRNA levels in dominant and recessive mutations at the mouse brown (b) locus

Characterization of TRP-1 mRNA Levels in Dominant and Recessive Mutations at the Mouse *brown* (*b*) Locus

Ian J. Jackson,* Doreen Chambers,* Eugene M. Rinchik† and Dot C. Bennett‡

**MRC Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU, Scotland, †Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831–8077, and ‡Department of Anatomy, St. George's Hospital Medical School, London SW17 ORE, England*

Manuscript received March 22, 1990
Accepted for publication June 20, 1990

ABSTRACT

The mouse *brown* locus encodes a putative membrane-bound metalloenzyme, tyrosinase-related protein-1 (TRP-1). We have examined the effect on mRNA expression of the locus of a number of mutant alleles. The common null mutant allele, *brown*, produces wild-type levels of TRP-1 mRNA, which is nonfunctional. Another recessive allele, *cordovan-Harwell*, has an intermediate, dark-brown phenotype and produces only very low levels of presumably normal TRP-1 mRNA. Two dominant alleles appear to act by killing the melanocyte in which they are expressed. One of them, *Light*, has normal size and amounts of TRP-1 mRNA. The other, *White-based brown*, produces no detectable TRP-1 mRNA. It has a gross DNA rearrangement at the 5′ end, and we speculate that this results in activation of transcription of sequences not usually seen in melanocytes, and that this is toxic to the cell. The relationship between phenotype and molecular structure at the locus is discussed, and we draw some general principles applicable to other developmental genes.

THE formation of the mouse coat color is a developmental system particularly amenable to molecular genetic study (SILVERS 1979; JACKSON 1985). Pigmentation is not essential to the viability of the laboratory mouse, and new mutations will therefore generally survive. Furthermore, coat color is a particularly striking characteristic, thus new mutations are readily identified. A large number of mutations at many loci have been found to affect coat color. Six loci in particular [*non-agouti* (*a*), *brown* (*b*), *albino* (*c*), *dilute* (*d*), *pink-eyed dilution* (*p*) and *piebald* (*s*)] have been used for many years in specific-locus mutation experiments, which have produced many new alleles at these loci (RUSSELL 1951; SEARLE 1974). Some mutations affect only pigment or pigment-related functions, while others have marked developmental effects, usually due to deletions of DNA associated with the locus (RUSSELL 1971; RUSSELL, MONTGOMERY and RAYMER 1982).

The cDNA corresponding to the product of the murine chromosome 7 *c* locus, tyrosinase, has been cloned (KWON *et al.* 1987; YAMAMOTO *et al.* 1987; MULLER *et al.* 1988). We have recently shown that a different cDNA clone, known as pMT4 (SHIBAHARA *et al.* 1986), encodes another protein, tyrosinase-related protein-1 (TRP-1), which shares approximately 40% amino acid identity with tyrosinase, and maps to the *b* locus on chromosome 4 (JACKSON 1988). Another tyrosinase-related protein (TRP-2) has been identified (called clone 5A), but not as yet mapped (JACKSON 1988; I. J. JACKSON, unpublished results). These three proteins have several common features. All have a hydrophobic residue near to the C terminus, which is probably a transmembrane domain (it is known that tyrosinase is a membrane-bound enzyme) [see HEARING and JIMENEZ (1989) for example]. All three also have striking similarity to tyrosinases of lower eukaryotes (*Neurospora crassa*) and of prokaryotes (*Streptomyces* species) in two particular domains (LERCH, LONGONI and JORDI 1982; HUBER, HINTERMAN and LERCH 1985; MULLER *et al.* 1988). One of these regions is also remarkably similar to the copper-binding domain of hemocyanins, particularly in conservation of the histidine residues (GAYKAMA *et al.* 1984; HUBER, HINTERMAN and LERCH 1985). Neurospora and Streptomyces tyrosinases are known to contain two copper atoms per molecule (SOLOMON 1981; HUBER, HINTERMAN and LERCH 1985). In the mouse it would appear that there are at least three membrane-bound, copper-containing enzymes, which are most likely localized on the inner membrane faces of the melanosomes, the site of pigment synthesis within the melanocyte.

The tight linkage we have reported of the TRP-1 probe to *brown* merely suggests, but does not prove, allelism. Recently we have shown sequence differences in the TRP-1 mRNA which are causative of the *brown* phenotype (ZDARSKY, FAVOR and JACKSON 1990). In this study, we find changes in TRP-1 mRNA expression and in TRP-1 gene structure in certain *b*- locus mutants, which provide additional evidence of allelism and allow an explanation of the phenotypes.

Mice doubly heterozygous for certain radiation-induced *brown* mutations are fully viable but have complete deletions of the TRP-1 locus and have a brown phenotype indistinguishable from the classical *brown* (E. M. RINCHIK unpublished results). Brown is therefore the null phenotype and results in melanocytes containing brown eumelanin instead of the wild-type black. Previously, examination of *brown* melanocytes led to the suggestion that its gene product played a role in melanosome morphology (RITTENHOUSE 1968). The sequence of TRP-1, however, would lead us now to propose that it is a membrane-bound metalloprotein that has some enzymatic function and shares a common ancestry with tyrosinase. While null mutations of tyrosinase (the *c* locus) cause complete absence of pigment, this is not the case at the *b* locus. The function of wild-type TRP-1 is therefore one that is not essential for pigment synthesis, but is necessary for black rather than brown pigment to be formed. The enzymology of melanin synthesis is not well understood, but there are a number of candidate enzymes which could be represented by TRP-1 (KORNER and PAWALEK 1980, 1982; BARBER *et al.* 1984).

In this work we have examined expression of TRP-1 mRNA from a number of alleles at the *b* locus. This locus is unusual among pigmentation genes as it has mutant alleles both recessive and dominant to wild type (*Black*). The recessive alleles result in production of either brown or dark-brown eumelanin. The two dominant alleles, *Light* (B^{lt}) and *White-based brown* (B^{w}), result in the tips of the hairs being pigmented, but the bases being much paler (MACDOWELL 1950; HUNSICKER 1969). Both are expressed when heterozygous with wild type (*Black*) but are more extreme in phenotype when homozygous. The phenotype appears to be due to a "suicide activation" of the *b* locus, resulting in death, or failure to remain in the hair bulb, late in the hair cycle, of those melanocytes which are undertaking pigment synthesis. In *Light* mice, at each hair growth cycle, pigment is made for a time, before the cells begin to die and are incorporated into the hair shaft. By the end of the growth cycle there are no melanocytes visible in the hair bulb (QUEVEDO and CHASE 1958; SWEET and QUEVEDO 1968; QUEVEDO, FLEISCHMANN and DYCKMAN 1981). New growths of hair, following molting, have pigmentation restored, but successive rounds of hair growth have less pigment at the hair tip, possibly reflecting a decreasing pool of melanocyte precursor cells on which to draw or, less likely, a more rapid suicide of newly recruited melanocytes (perhaps due to their having a low level of *b* locus transcription before recruitment).

Table 1 summarizes the mutants examined in this study, their origin and their phenotypes. We report the characterization of expression of the alleles and present the basis for detailed further molecular analysis of the relationship between genotype and phenotype at the *b* locus.

MATERIALS AND METHODS

Mice and cell lines: C57BL/6J, *cordovan-Harwell* and *Light* mice are maintained at the Animal Unit of the Western General Hospital. *White-based brown* mice are maintained at the Biology Division of Oak Ridge National Laboratory.

The B16C3 melanoma cells were obtained originally from J. KREIDER and grown as described previously (BENNETT 1983). Melan-a and melan-b lines were described by BENNETT, COOPER and HART (1987) and BENNETT *et al.* (1989) and grown as described.

Hybridization probes: Most hybridizations used the 1.6-kb internal *Hin*dIII fragment of pMT4, recloned into *Bluescribe* (Stratagene) (JACKSON 1988; SHIBAHARA *et al.* 1986). This fragment detects the diagnostic D- and B-haplotype fragments, in addition to several others. To examine the 5′ end of TRP-1 an 800-bp *Eco*RI to *Pst*I fragment from a genomic clone of the TRP gene was used. The TRP-2 probe was the 1200-bp *Eco*RI fragment of clone 5A (JACKSON 1988), subcloned into *Bluescribe*. Actin mRNA was detected using the cDNA from clone pAM91 (MINTY *et al.* 1981) The fragments were isolated from low gelling temperature agarose, diluted with 3 volumes of water and labeled with ^{32}P using the method of FEINBERG and VOGELSTEIN (1983).

DNA methods: DNA was made by homogenization of organs in STE [100 mM NaCl, 50 mM Tris-HCl (pH 8), and 10 mM EDTA], followed by treatment with proteinase K (100 μg/ml) in 0.5% sodium dodecyl sulfate (SDS) for several hours. After phenol extraction and chloroform extraction the DNA was precipitated with ethanol and dissolved in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA.

Restriction digestions were performed using manufacturers' recommended buffers.

DNA was electrophoresed through 0.8% agarose in TAE [40 mM Tris-acetate (pH 7.5) and 1 mM EDTA] and transferred to Hybond-N (Amersham) nylon filters according to manufacturer's instructions.

Southern blot hybridizations were carried out in 3 × SSC, 10 × Denhardt's solution supplemented with 100 μg/ml denatured salmon sperm DNA, 0.1% SDS and 0.1% sodium pyrophosphate (JEFFREYS and FLAVELL 1977) [1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate (pH 7) and 1 × Denhardt's solution is 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% and polyvinylpyrridoline] or in 0.5 M sodium phosphate (pH 7.2), 7% SDS (CHURCH and GILBERT 1984) and washed at 68° down to 0.1 × SSC and 0.1% SDS before autoradiography.

RNA methods: RNA was made from fresh or frozen melanoma or melanocyte cell pellets, and from fresh or frozen dorsal skin of 1–4-day-old mice. Cells or skin were homogenized in 3 M LiCl, 6 M urea (LOVELL-BADGE 1987), the RNA precipitated overnight, centrifuged and washed in homogenization buffer. After resuspension in 10 mM Tris-HCl (pH 7.5) and 0.1% SDS, the RNA was digested with proteinase K at 100 μg/ml at 65° for several hours, phenol extracted, chloroform extracted, and ethanol precipitated.

The RNA was electrophoresed through 1% agarose gels containing 2.2 M formaldehyde in 40 mM MOPS (pH 7), 5 mM sodium acetate and 1 mM EDTA (LEHRACH *et al.* 1977) before blotting in 10 × SSC to nitrocellulose filters.

Northern-blot hybridizations were carried out in 50% formamide, 4 × SSC, 10 × Denhardt's solution, 50 mM sodium phosphate, 100 μg/ml salmon sperm DNA and 0.1% SDS overnight at 48° and washed down to 0.3 × SSC at 68° before autoradiography.

TABLE 1

Alleles at the *brown* locus

Allele	Symbol	Phenotype	Origin
Black	*B*	Black eumelanin	Wild type
brown	*b*	Brown eumelanin	Mouse fancy
cordovan-Harwell	b^{cH}	Dark-brown eumelanin	Radiation mutagenesis
Light	B^{lt}	Dominant; hair tip more pigmented than base, and is black or dark-brown	Spontaneous
White-based brown	B^{w}	Dominant; similar to *Light* but more extreme, and pigment is brown	Radiation mutagenesis

Reverse transcription and the polymerase chain reaction: Double-stranded cDNA was prepared using a kit supplied by Boehringer Mannheim Ltd., according to the manufacturer's instructions, except that oligo(dT) primer was replaced with 36 μg/ml random hexanucleotides (dN_6) (Pharmacia).The oligonucleotide primers used for the polymerase chain reaction (PCR) were: TCCGAATTCAAAGGGGTGGATGACCG (bases 293 to 312 plus a terminal *Eco*RI site) and GACACATAGTAATGCATCC (633 to 615) from the tyrosinase cDNA (MULLER *et al.* 1988); CAATTAACAGCTGGCATCA (−175 to −157) and GGAAGGTTTCTCTGCTGA (−97 to −114) from the 5′ untranslated region of TRP-1 cDNA; and GCTGCAGGAGCCTTCTTTC (714 to 732) and GACGCTGCACTGCTGGTCT (961 to 979) from the middle of TRP-1 cDNA (SHIBAHARA *et al.* 1986).

The reactions were performed essentially as in SAIKI *et al.* (1988). Each reaction contained 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.01% NP-40, all six oligonucleotide primers at 3 μg/ml, all four deoxynucleotide triphosphates at 0.1 mM and 2.5 units Taq DNA polymerase (Amersham) in a volume of 50 μl. Some reaction mixes were irradiated with UV by placing on a UV Products transilluminator for 10 min before addition of enzyme and substrate (SARKAR and SOMMER 1990). Each cDNA amplification used the product of reverse transcription of approximately 0.1 μg skin total RNA. The reaction was cycled 25 times through 90 sec at 92°, 90 sec at 53° and 120 sec at 72°. Thirty percent of the reaction was analyzed by electrophoresis on gels of 4% Nusieve GTG agarose (FMC Bioproducts) for ethidium bromide staining, or of 1.5% agaraose (Sigma) for Southern blotting.

RESULTS AND DISCUSSION

Both TRP-1 and TRP-2 are melanocyte-specific, highly expressed mRNAs. Northern blot analysis on a range of tissues reveals no evidence of expression in cells other than melanocytes and melanoma. *In situ* hybridization to sections of neonatal mouse skin shows that TRP-1 is expressed exclusively in the melanocytes of the hair follicles (K. STEEL, D. R. DAVIDSON and I. J. JACKSON, unpublished results).

SHIBAHARA *et al.* (1986) reported two discrete TRP-1 mRNA species visible on Northern blots. Although the existence of two species is demonstrated by cDNA clones differing at their 3′ ends (SHIBAHARA *et al.* 1986), when blots are washed at high stringency we see only a single species (see Figures 1, 2 and 3, for example), although additional bands can be seen at lower stringency. The high abundance of TRP-1 mRNA in melanocytes permits its detection in neonatal skin samples, where melanocytes comprise only a small minority of cells. Approximately the same signal is obtained on a Northern blot of 10 μg of 2-day-old skin total RNA, as from 100 ng of melanoma total RNA. Hybridization of TRP-2 cDNA to Northern blots reveals a slightly smaller mRNA, which, in this study, we use as a control, not only for the integrity and loading of RNA, but also to confirm the presence of melanocytes in the skin samples examined.

The *brown* (*b*) mutation: The common, recessive *brown* (*b*) mutation is an old mutation of the mouse fancy. We have shown (JACKSON 1988) that the mutation present in a number of different laboratory inbred and outbred stocks is associated with a 4.9-kb *Taq*I TRP-1 fragment, the D haplotype. All other mice have the B haplotype comprising a 3.7- and 1.2-kb *Taq*I fragment.

Figure 1 shows autoradiography of a Northern blot, probing total RNA made from melanoma cells and melanocytes grown *in vitro*. B16 melanoma cells and melan-a cells (BENNETT, COOPER and HART 1987) are both derived from C57BL/6 inbred mice, which are wild type (*Black*) at the *b* locus. Melan-b cells (BENNETT *et al.* 1989) are derived from homozygous *brown* mice of the outbred Q stock. After washing to remove the first probe, the filter was rehybridized with TRP-2 to check for loading. There is no signficant difference in the abundance or size of TRP-1 mRNA between *Black* and *brown* melanocytes, or between melanoma and cultured melanocytes. The Q-stock *brown* mutation is associated with the D haplotype (JACKSON 1988), and this result is therefore most likely applicable to the common *brown* mutation seen in all laboratory mice.

The defect which results in the brown phenotype is not one of transcription or processing, but must be a small change, probably a point mutation, which renders the TRP-1 mRNA or its protein product inactive. We have recent sequencing evidence that there are two amino acid differences between the TRP-1 product of *Black* and *brown* mice, and can show that only one of these; Cys-86 to tyrosine, results in the mutant

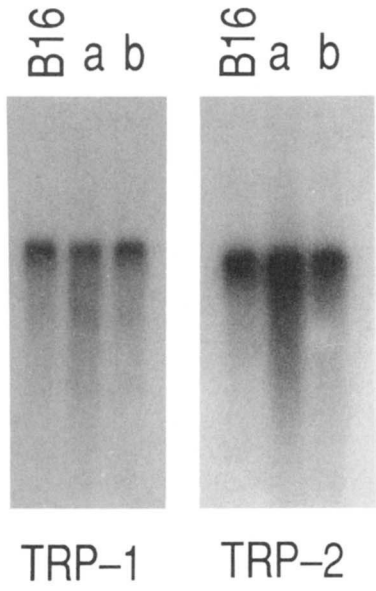

FIGURE 1.—Northern blot hybridization to total RNA from B16 melanoma cells (B16), melan-a melanocytes (a) and melan-b melanocytes (b). The filter was hybridized first with the 1.6-kb *Hin*dIII fragment of pMT4 and exposed to film to reveal the 2500-nucleotide TRP-1 mRNA before washing to remove the first probe and rehybridization with the 1.2-kb *Eco*RI fragment of clone 5A to show the 1900-nucleotide TRP-2 mRNA.

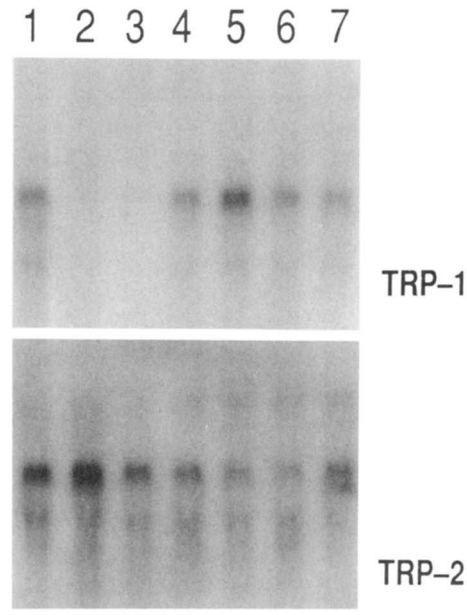

FIGURE 2.—Northern blot hybridization to total RNA prepared from dorsal skin of each member of a 2-day-old litter. The filter was hybridized as Figure 1; first to show TRP-1 (upper panel), secondly to show TRP-2 (lower panel). Genotypes, determined by Southern blot analysis of *Taq*I-digested DNA, are: animal 5, b/b; animals 1, 4, 6 and 7, b^{cH}/b; animals 2 and 3, b^{cH}/b^{cH}.

phenotype (ZDARSKY, FAVOR and JACKSON 1990).

The *cordovan-Harwell* (b^{cH}) mutation: A number of alleles of *brown* have been described that are intermediate in phenotype between black and brown. They have been variously called dark-brown or cordovan. The first intermediate allele to be described, called *cordovan* (b^{c}), arose spontaneously (MILLER and POTAS 1955). We have examined transcription of another intermediate allele, *cordovan-Harwell* (b^{cH}), which arose in a γ-irradiation mutagenesis experiment in a (C3H/HeH × 101/H)F_1 male receiving approximately 600 rad (6 Gy) over a period of about 12 weeks (BATCHELOR, PHILLIPS and SEARLE 1966).

We have examined mRNA levels of the b^{cH} allele in RNA prepared from neonatal skin of litters segregating b^{cH} and b. It is not possible to distinguish mice homozygous for *cordovan-Harwell* from those heterozygous with *brown* (*i.e.*, the mutation is fully dominant over *brown*). Furthermore it is difficult to distinguish these mice from homozygous *brown* animals when they are only a few days old. However, as the b^{cH} mutation arose on mice carrying the B haplotype, Southern blots of DNA from these neonates allow distinction to be made between animals homozygous for either B or D haplotypes (b^{cH} or b, respectively), or compound heterozygotes (b/b^{cH}).

We prepared RNA from the skin and DNA from the kidneys of each member of a 2-day-old litter derived from parents that were compound heterozygotes, b/b^{cH}. Analysis of the DNA by Southern blot hybridization to *Taq*I digests determined the genotype of each member of the litter. Figure 2a is an autoradiograph of a Northern blot of the skin RNAs probed with pMT4. TRP-2 was hybridized subsequently to confirm the RNAs were undegraded and equally loaded (Figure 2b). Animal 5 is the only b/b animal of the litter. As the mRNA abundance is the same in *Black* (+/+) or *brown* (b/b) melanocytes (see above), this animal serves as an indicator of wild-type TRP-1 mRNA level. Animals 2 and 3 are both homozygous b^{cH}/b^{cH}, and the rest are b/b^{cH}. Comparison of the hybridization signals, within the litter and with the control hybridization to TRP-2, shows that the homozygous *cordovan-Harwell* mice have very low levels of TRP-1 mRNA. The heterozygotes have about 50% wild-type amount of mRNA, largely due to the *brown* (b) allele. Hybridization to TRP-1 in the b^{cH}/b^{cH} skin is detectable in lanes 2 and 3, but we estimate from scanning the autoradiograph that it is present at approximately 1% of the abundance of b/b or +/+ mice. As the phenotype of *cordovan-Harwell* is not brown, but is somewhat darker, we would expect the melanocytes to have reduced, but not absent, TRP-1 activity. It therefore is likely that the low level of TRP-1 seen represents normal mRNA, and that the phenotype is due to a greatly reduced level of its (normal) protein product.

The effect of dosage of TRP-1 on pigmentation is put into an interesting light by the mutation, and is summarized in Table 2. If we make the reasonable assumption that relative protein levels correspond to the observed mRNA levels, we can propose that al-

TABLE 2

Dosage of TRP-1 and quantized phenotype

Genotype	Observed mRNA hybridizing (%)	Approximate level of presumed active mRNA (%)	Phenotype
B/B	100	100	Black
B/b	100	50	Black
b^{cH}/b^{cH}	1	1	Dark brown
b^{cH}/b	50	0.5	Dark brown
b/b	100	0	Brown

Assuming that *brown* TRP-1 mRNA is fully inactive, and *cordovan-Harwell* TRP-1 mRNA is fully active.

though complete lack of active protein (*b/b*) leads to a brown phenotype, half normal levels (*B/b*) results in a phenotype indistinguishable from wild type (*B* is dominant over *b*). A reduction to about 1% wild-type levels of TRP-1 in b^{cH}/b^{cH} mice leads to an clear change in pigmentation from black toward brown, but further reduction to about 0.5% (b^{cH}/b) has no further effect (b^{cH} is dominant over *b*). Two explanations present themselves. First the effect of TRP-1 on pigment synthesis might show a threshold or quantized effect, meaning that there exist discrete forms of pigment, governed by a particular dose of TRP-1, rather than a continuum of pigment quality between black and brown. Alternatively, such quantization might be perceptual; there may be a continuum, but we cannot perceive the difference between more than a limited number of states. A different situation occurs at the *albino* (*c*) locus, at which many intermediate alleles [such as *chinchilla* (c^{ch})], are recessive to wild type but only semidominant over *albino* (so, for example, c^{ch}/c^{ch} mice are darker than c^{ch}/c) (SILVERS 1979). The difference between the *b* and *c* loci might be due to the function of their products. Tyrosinase, from the *c* locus regulates the amount of pigment produced while the *b*-locus product, TRP-1, governs the quality (or possibly stability) of the pigment color.

The molecular basis of the reduced TRP-1 mRNA abundance is currently under investigation. The nature of the change will be informative as to the regulation of mRNA levels in general and in melanocytes in particular. The reduction may be due to decreased transcription or increased degradation of the RNA or its precursor. It should be noted that we have not been able to detect by restriction fragment analysis any difference between this mutant TRP-1 gene and the wild-type allele, even though the mutation arose following γ-irradiation.

The dominant *Light* (B^{lt}) mutation: *Light* (B^{lt}) is the better studied of the dominant alleles of *brown* (QUSEVEDO and CHASE 1958; SWEET and QUEVEDO 1968; QUEVEDO, FLEISCHMANN and DYCKMAN 1981). It arose spontaneously on a C58 background, and Southern blot examination of DNA from homozygous *Light* mice shows no evidence of alteration of gene structure. We have examined the TRP-1 mRNA level in the skin of homozygous B^{lt} neonates (whose genetic status was confirmed by haplotype analysis) compared with age-matched C57BL/6 (wild-type) animals. At this early stage of the hair cycle we would not yet expect to see a depletion in melanocyte numbers. Figure 3 shows a Northern blot of these RNAs probed with the pMT4 plasmid, which indicates that there is no difference in TRP-1 mRNA size or abundance.

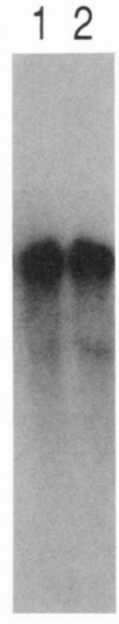

FIGURE 3.—Northern blot hybridization to total RNA prepared from dorsal skin of a 3-day-old C57BL/6 mouse (*B/B*, *i.e.* +/+) (lane 1) and a 3-day-old homozygous *Light* mouse (B^{lt}/B^{lt}) (lane 2). The filter was hybridized to detect TRP-1 as described in the text.

We conclude that the *Light* mutation most likely exerts its effect through a mutant protein which causes the observed death of the melanocytes. This protein probably has some residual, normal TRP-1 activity, as the pigment produced by homozygous mutant melanocytes is dark brown rather than brown, but, in addition the protein must be toxic to the cell. Either it is itself toxic (through mislocalization in the melanocyte, for example), or its enzymatic function has been altered so that a toxic product accumulates in the cell.

The dominant *White-based brown* (B^{w}) mutation: The phenotype of *White- based brown* (B^{w}) mice is very similar to that of *Light*, although it has not been studied in the same detail. We propose that the product of the B^{w} allele, like that of B^{lt}, causes melanocyte death, but, unlike B^{lt}, homozygous B^{w}/B^{w} mice, or compound heterozygotes with *brown* (B^{w}/b), have brown pigment at the hair tips. Thus the mutation results in a recessive null phenotype (brown) in addition to the dominant (white-base) phenotype. The mutation arose in a spermatogonium of a (101/Rl x C3H/Rl)F_1 male, exposed to 600 rad (6 Gy) of γ-radiation over a period of several weeks (HUNSICKER 1969).

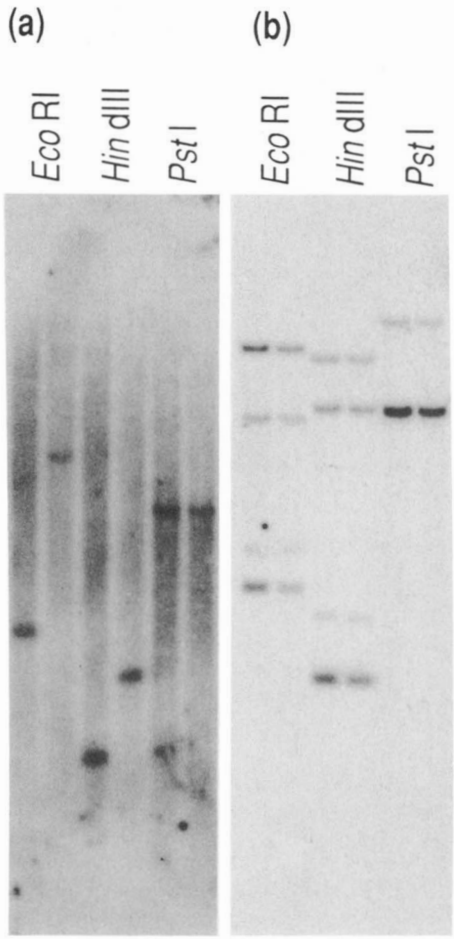

FIGURE 4.—Southern blot hybridization to DNA prepared from C3H/He mice and from homozygous *White-based brown* (B^w/B^w) mice. In each pair of tracks, digested with the restriction enzyme indicated above, the left-hand track is *White-based brown* DNA and the right-hand track is C3H/He DNA. Panel a was hybridized with the 800-bp *Eco*RI to *Pst*I genomic clone fragment and panel b is the same filter rehybridized with the 1.6-kb *Hin*dIII cDNA fragment.

Figure 4 shows this allele is associated with a DNA rearrangement detected by TRP-1 cDNA and genomic probes. The mutation arose in a hybrid mouse, which has two different chromosomes *4*, but we do not know on which chromosome it occurred. We have compared DNA restriction fragments from homozygous B^w mice with C3H DNA, but we observe essentially identical fragments when compared with 101 DNA (with the exception of variants in an unlinked fragment cross-hybridizing with the 3′ end). Probes encompassing most of the coding region of the TRP-1 gene do not detect any differences between the B^w allele and the C3H wild-type gene (Figure 4b). However, when probes toward the 5′ end of TRP-1 are used, differences are found between the two genes.

The 5′ end of the wild-type gene is enclosed within an 800-bp *Eco*RI-*Pst*I fragment. The *Eco*RI site is about 100 bp upstream of the 5′ end of the mRNA. There is an intervening sequence beginning after base −87 of the cDNA sequence (*i.e.*, 99 bp from the end

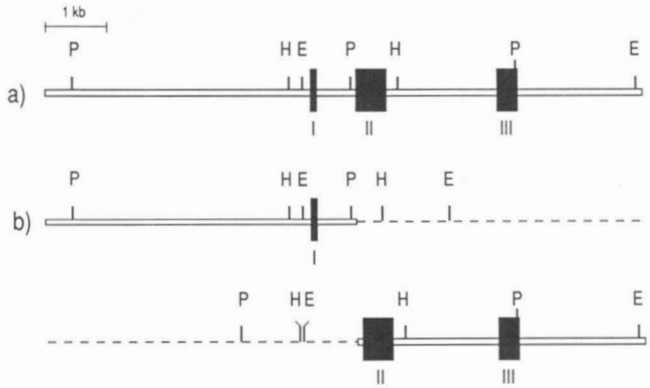

FIGURE 5.—Schematic representation of the 5′ ends of the wild-type and B^w mutant TRP-1 genes. (a) Wild-type gene, showing first 3 exons, numbered I, II and III. (b) B^w mutant gene. Novel DNA sequences are represented by dashed lines. It is not known if these sequences are a single insertion into the locus, or are flanking a site of a large inversion. Both halves of the rearrangement are shown. P, *Pst*I; E, *Eco*RI; H, *Hin*dIII. Not all sites are shown.

of the published sequence) and the *Pst*I site is approximately 600 bp into this intron (see Figure 5a). This *Eco*RI-*Pst*I fragment, containing exon I, was used to probe Southern blots of DNA from homozygous *White-based brown* and wild-type animals and the results are shown in Figure 4a. When digested with *Pst*I, the B^w/B^w DNA has the same fragment size as wild type (4.5 kb), indicating that sequences 5′ of the *Pst*I site in the first intron are unrearranged (which include a *Hin*dIII and an *Eco*RI site). However, the 6.5-kb *Eco*RI wild-type fragment is replaced in *White-based brown* DNA by one of 2.4 kb, and the 2.15 kb *Hin*dIII fragment replaced by one of 1.3 kb. There is therefore a DNA rearrangement within the B^w TRP-1 gene, downstream of the *Pst*I site in the first intron, but upstream of the *Hin*dIII and *Eco*RI sites, which are in the second and third introns, respectively. As the novel *Hin*dIII site lies only 0.2 to 0.3 kb downstream of the (unaffected) *Pst*I site, the rearrangement must have an end point in this few hundred base-pair interval. Other data (not shown) indicates the breakpoint lies close to the 3′ end of the first intron.

The exact nature of the rearrangement has to be established, but it involves a juxtaposition of novel DNA sequences with the 5′ end of the gene. The data are consistent with an insertion of DNA at this point, or an inversion of the region with one breakpoint mapping to end of the intron, and the other an unknown distance upstream. Figure 5 is a schematic representation of the first 3 introns of the wild-type TRP-1 gene, and both halves of the rearrangement in the B^w mutation, and includes additional mapping data.

We are unable to detect transcription from the B^w mutant allele. Figure 6 shows the results of probing a Northern blot of RNA made from 2-day-old wild-type (C57BL/6) skin (lanes 1, 3, 5 and 7) and from 2-day-

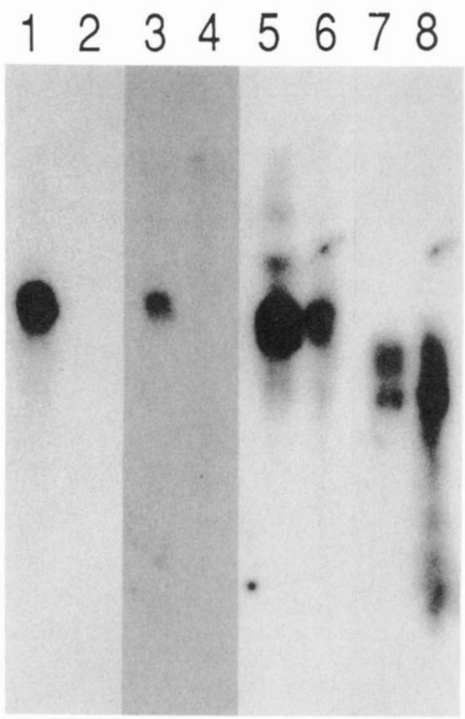

FIGURE 6.—Northern blot hybridization to total RNA prepared from dorsal skin of a 2-day-old C57BL/6 mouse (*B/B, i.e.* +/+) (lanes 1, 3, 5 and 7) and 2-day-old homozygous *White-based brown* (B^w/B^w) mice (lanes 2, 4, 6 and 8). The filter was hybridized as in Figure 1, first to detect TRP-1 downstream of the rearrangement (lanes 1 and 2), then to detect TRP-1 transcripts upstream of the rearrangement (lanes 3 and 4), subsequently TRP-2 (lanes 5 and 6) and finally actin (lanes 7 and 8).

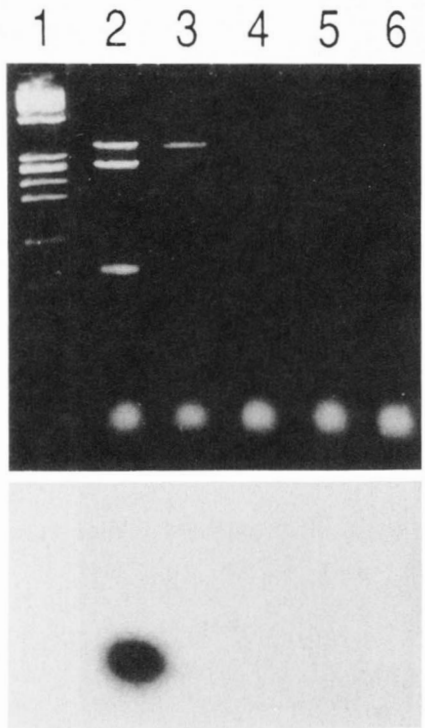

FIGURE 7.—Gel analysis of PCR-amplifications of dorsal skin cDNA. Upper panel: ethidium bromide stained 4% Nu-Sieve agarose gel. Lane 1, *Hae*III-digested ϕX174 DNA as size markers; lane 2, amplification products of wild-type (C57BL/6) cDNA; lane 3, products of homozygous B^w/B^w cDNA; lane 4, amplification of C57BL/6 RNA as control; lane 5, amplification of B^w/B^w RNA as control; lane 6, control amplification with no substrate. Amplified fragment sizes in track 2 are 348, 266 and 78 bp. Lower panel: Southern blot hybridization of a similar reaction run on a 1.5% agarose gel, probed with the 800-bp *Eco*RI-*Pst*I (exon I) fragment of TRP-1. Lanes as above. The autoradiograph shows hybridization only to the 78-bp exon I PCR product of wild-type cDNA.

old B^w/B^w skin (lanes 2, 4, 6 and 8). When the blot is probed either with the 1600 bp cDNA fragment from downstream of the rearrangement (lanes 1 and 2) or with the *Eco*RI/*Pst*I genomic fragment containing the first exon (upstream of the rearrangement) (lanes 3 and 4) there is no evidence of a TRP-1-containing transcript from the B^w allele. Reprobing the blot with TRP-2 cDNA (lanes 5 and 6) results in hybridization to all tracks, indicating that the RNA from B^w mice is intact, and that there are indeed melanocytes present in the skin. The hybridization to TRP-2 mRNA is somewhat weaker in the B^w/B^w RNA than the wild type. This is not due to a lower RNA loading as reprobing with actin cDNA (lanes 7 and 8) shows that there is, in fact, more RNA in the mutant track. It is possible that we may be seeing here an early indication of the depletion of the melanocyte pool, which ultimately results in the phenotype.

It is not surprising that the DNA rearrangement results in no transcription from the main part of the gene. However, as it appears from Southern blotting that the 5′ flanking region is intact, it may be capable of driving transcription of the first exon, as well as sequences downstream including sequences within the rearrangement. Such transcripts may not be of discrete size, and may be hidden in the smear seen in track 4 of Figure 6. We therefore used the polymerase chain reaction (PCR) to examine homozygous B^w RNA. We made double-stranded cDNA from both wild-type and mutant skin RNA [priming the first strand with random hexanucleotides to allow for a possible lack of poly(A) tail]. This was then used as a substrate for the PCR (SAIKI *et al.* 1988), primed by oligonucleotide pairs from downstream of the rearrangement, from upstream (within the first exon) and, as control, from the tyrosinase gene. Figure 7 (top) shows an ethidium bromide-stained gel of the amplified DNA. Amplification of wild-type cDNA gives rise to a 348-bp fragment deriving from the tyrosinase mRNA, and to 266- and 78-bp fragments from TRP-1 mRNA (track 2). B^w cDNA gives rise only to the amplified tyrosinase fragment; TRP-1 transcripts from either side of the rearrangement are not visible (track 3). Figure 7 (bottom) is a Southern blot of a similar gel probed with exon 1, and shows at higher sensitivity that no exon 1 transcripts are present in B^w/B^w skin. We conclude that the TRP-1 promoter associated with the B^w allele most likely is nonfunctional due to disruption by the rearrangement.

White-based brown has a recessive brown phenotype which is obviously due to absence of any TRP-1 transcript. However, the mutation also has a true dominant effect, and therefore it is likely that a transcript derives from the region of the locus, and this transcript (or its translation product) causes melanocyte death. While it is possible that very low levels of a fusion transcript containing the first exon and other sequences might be the agent, this is perhaps unlikely. A better hypothesis is that the rearrangement has brought another gene (or genes), not normally expressed in melanocytes, into the proximity of the TRP-1 gene, which supplies it with enhancer (but not necessarily promoter) function. It might be the ectopic expression of this gene (either high levels of an aberrant RNA alone or the translation product of an ectopic mRNA) which results in the dominant phenotype. The nature of the DNA rearrangement in *White-based brown* mice, and the sequences encompassed by it are of great interest, and will be further characterized by DNA cloning.

GENERAL CONCLUSIONS

This study raises several issues that are generally applicable and should be borne in mind when other developmental mutations are considered. First, phenotype can be quantized in addition to the usually observed dominance of wild-type over null mutation. Second, simple mutations in highly expressed enzymes can lead to neomorph dominant functions which can have profound effects on cell function or survival. Furthermore it seems that a mutation involving only a small change (B^{lt}) can have a very similar phenotypic effect to one resulting from a much more severe DNA disruption (B^{w}). The mode of action of these dominant, cell-suicide, alleles is of general relevance to developmental genetics; cell-type specific, autonomous cell death might well be the basis of many developmental mutations which have profound effects on morphology. Finally, it should be noted that two mutations examined here arose in γ-irradiation experiments, and both received approximately the same dose (600 rad or 6 Gy) over approximately the same time period, but only in one have we detected a gross DNA rearrangement. However, both mutations were selected on the basis of being distinguishable from *brown*, and so are not representative of the large number of other *brown* alleles obtained by radiation mutagenesis.

We thank NICK HASTIE and RUTH JOHNSON for useful discussion and encouragement, EMAN ZDARSKY for developing the RT-PCR methodology, LINDA DEVLIN for skilled assistance and NICK HASTIE, JOHN EVANS and RUTH JOHNSON for comments on the manuscript. NORMAN DAVIDISON, DOUGLAS STUART and SANDY BRUCE provided excellent photographic and art work. We also thank the Medical Research Council (MRC) and the Lister Institute for Preventive Medicine for financial support. The work has been funded in part by an MRC Project Grant to I.J.J., by the Office of Health and Environmental Research, U.S. Department of Energy, under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems Inc. (E.M.R.) and by a CRC grant to D.C.B. I.J.J. is a Lister Fellow.

LITERATURE CITED

BARBER, J. I., D. TOWNSEND, D. P. OLDS and R. A. KING, 1984 DOPAchrome oxidoreductase: a new enzyme in the pigment pathway. J. Invest. Dermatol. **83:** 145–149.

BATCHELOR, A. L., R. J. S. PHILLIPS and A. G. SEARLE, 1966 A comparison of the mutagenic effectiveness of chronic neutron and gamma-irradiation of mouse spermatogonia. Mutat. Res. **3:** 218–229.

BENNETT, D. C., 1983 Differentiation in mouse melanoma cells; initial reversibility and an on-off stochastic model. Cell **34:** 445–453.

BENNETT, D. C., P. J. COOPER and I. R. HART, 1987 A line of non-tumorigenic mouse melanocytes, syngeneic with the B16 melanoma and requiring a tumour promoter for growth. Int. J. Cancer **39:** 414–418.

BENNETT, D. C., P. J. COOPER, T. J. DEXTER, L. M. DEVLIN, J. HEASMAN and B. NESTER, 1989 Cloned mouse melanocyte lines carrying the germline mutations albino and brown. Development **105:** 379–385.

CHURCH, G. M., and W. GILBERT, 1984 Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:** 1991–1995.

FEINBERG, A. P., and B. VOGELSTEIN, 1983 A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:** 6–13.

GAYKAMA, W. P. J., W. G. H. HOL, J. M. VEREIJKEN, N. M. SOETER, H. J. BAK and J. J. BEINTEMA, 1984 3.2 Angstrom structure of the copper-containing oxygen-carrying protein *Panulirus interruptus* haemocyanin. Nature **309:** 23–29.

HEARING, V. J., and M. JIMENEZ, 1989 Analysis of mammalian pigmentation at the molecular level. Pigment Cell Res. **2:** 75–85.

HUBER, M., G. HINTERMAN and K. LERCH, 1985 Primary structure of tyrosinase from *Streptomyces glaucesens*. Biochemistry **24:** 6038–6044.

HUNSICKER, P. R., 1969 Mouse News Lett. **40:** 41.

JACKSON, I. J., 1985 The genetics and biology of mouse melanocytes; mutation, migration and interaction. Trends Genet. **1:** 321–326.

JACKSON, I. J., 1988 A cDNA encoding tyrosinase-related protein maps to the mouse brown locus. Proc. Natl. Acad. Sci. USA **85:** 4392–4396.

JEFFREYS, A. J., and R. FLAVELL, 1977 A physical map of the DNA region flanking the rabbit beta-globin gene. Cell **12:** 429–439.

KORNER, A. M., and J. M. PAWALEK, 1980 Dopachrome conversion: a possible control point in melanin biosynthesis. J. Invest. Dermatol. **75:** 192–195.

KORNER, A. M., and J. M. PAWALEK, 1982 Mammalian tyrosinase catalyses three reactions in the biosynthesis of melanin. Science **217:** 1163–1165.

KWON, B. S., A. K. HAQ, S. H. POMERANTZ and R. HALABAN, 1987 Isolation and sequence of a cDNA clone for human tyrosinase that maps at the mouse c-albino locus. Proc. Natl. Acad. Sci. USA **84:** 7473–7477.

LEHRACH, H., D. DIAMOND, J. M. WOZNEY and H. BOEDTKER, 1977 RNA molecular weight determination by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16:** 4743–4751.

LERCH, K., C. LONGONI and E. JORDI, 1982 Primary structure of tyrosinase from *Neurospora crassa*. J. Biol. Chem. **257:** 6408–6413.

LOVELL-BADGE, 1987 Introduction of DNA into embryonic stem

cells, pp. 153–182 in *Teratocarcinomas and Embryonic Stem Cells; A Practical Approach*, edited by E. J. ROBERTSON. IRL Press, Oxford.

MACDOWELL, E. C., 1950 "Light," a new mouse colour. J. Hered. **41:** 35–36.

MILLER, D. S., and M. Z. POTAS, 1955 Cordovan, a new allele of black and brown color in the mouse. J. Hered. **46:** 293–296.

MINTY, A. J., M. CARAVATTI, B. ROBERT, A. COHEN, P. DAUBAS, A. WEYDART, F. GROS and M. E. BUCKINGHAM, 1981 Mouse actin messenger RNAs. Construction and characterization of a recombinant plasmid molecule containing a cDNA transcript of mouse alpha actin mRNA. J. Biol. Chem. **256:** 1006–1014.

MULLER, G., S. RUPPERT, E. SCHMID and G. SCHUTZ, 1988 Functional analysis of alternatively spliced tyrosinase gene transcripts. EMBO J. **7:** 2723–2730.

QUEVEDO, W. C., and H. B. CHASE, 1958 An analysis of the Light mutation of coat color in mice. J. Morphol. **102:** 329–345.

QUEVEDO, W. C., R. D. FLEISCHMANN and J. DYCKMAN, 1981 Premature loss of melanocytes from hair follicles of light (B^{lt}) and silver (*si*) mice, pp. 177–183 in *Pigment Cell 1981; Phenotypic Expression in Pigment Cells*, edited by M. SEIJI. University of Tokyo Press, Tokyo.

RITTENHOUSE, E., 1968 Genetic effects on fine structure and development of pigment granules in mouse hair bulb melanocytes. I. The b and d loci. Dev. Biol. **17:** 351–365.

RUSSELL, L. B., 1971 Definition of functional units in a small chromosomal segment of the mouse and its use in interpreting the nature of radiation induced mutations. Mutat. Res. **11:** 107–123.

RUSSELL, L. B., C. S. MONTGOMERY and G. D. RAYMER, 1982 Analysis of the albino locus region of the mouse. IV. Characterization of 34 deficiencies. Genetics **100:** 427–453.

RUSSELL, W. L., 1951 X-ray induced mutation in mice. Cold Spring Harbor Symp. **16:** 327–336.

SAIKI, R. K., D. H. GELFAND, S. STOFFEL, S. J. SCHARF, R. HIGUCHI, G. T. HORN, K. B. MULLIS and H. A. ERLICH, 1988 Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:** 487–491.

SARKAR, G., and S. S. SOMMER, 1990 Shedding light on PCR contamination. Nature **343:** 27.

SEARLE, A. G., 1974 Mutation induction in mice. Adv. Radiat. Biol. **4:** 131–207.

SHIBAHARA, S., Y. TOMITA, T. SAKAKURA, C. NAGER, B. CHANDHURI and R. MULLER, 1986 Cloning and expression of cDNA encoding tyrosinase. Nucleic Acids Res. **14:** 2413–2427.

SILVERS, W. K., 1979 *The Coat Colors of Mice: A Model for Mammalian Gene Action and Interaction*. Springer, New York.

SOLOMON, E. I., 1981 Binuclear copper active site. Hemocyanin, tyrosinase and type 3 copper oxidases, pp. 43–108 in *Copper Proteins*, edited by T. G. SPIRO. Wiley, New York.

SWEET, S. E., and W. C. QUEVEDO, 1968 Role of melanocyte morphology in pigmentation of mouse hair. Anat. Rec. **162:** 243–254.

YAMAMOTO, H., S. TAKEUCHI, T. KUDO, K. MAKINO, A. NAKATA, T. SHINODA and T. TAKEUCHI, 1987 Cloning and sequencing of mouse tyrosinase cDNA. Jpn. J. Genet. **62:** 271–274.

ZDARSKY, E., J. FAVOR and I. J. JACKSON, 1990 The molecular basis of *brown*, an old mouse mutation, of an induced revertant to wild type. Genetics **126:** 443–449.

Communicating editor: R. E. GANSCHOW